SELECTIVE REGULATION OF ADENOVIRUS PRODUCTION

This invention was made with Government support under Grants 1RO1 AI41636, 1PO1 CA28146 awarded by the National Institutes of Health and with support from the Cystic Fibrosis Foundation under Grant CFF 2161. The Government has certain rights in the invention.

FIELD OF THE INVENTION

This invention relates to the field of adenovirus vectors and the regulation of viral particle production. One area of application is in the field of DNA delivery systems.

BACKGROUND OF THE INVENTION

Adenovirus is a common human DNA virus that naturally infects the airway epithelia as well as other tissues in the body. Adenovirus ("Ad") is a particularly useful virus as a human DNA delivery system for a number of reasons. First, the genetic organization of the virus and functions of many virus-encoded gene products have been characterized. Second, the Ad genome is easily manipulated in the laboratory, and recombinant virus are readily grown to high titers in cultured cells. Third, Ad has a wide host cell range, and recombinant Ad vectors have been used to efficiently infect multiple cell types in culture and in animals. Adenoviruses have been shown to infect a variety of tissues in animal studies including liver, kidney, muscle, respiratory, endothelial and nervous system. Diseases that affect these and other tissues

therefore are amenable to adenovirus-mediated gene therapy. Fourth, Ad has the ability to efficiently infect non-dividing differentiated cells in the animal, a major target for DNA delivery applications. Finally, adenovirus is a relatively benign human virus that is associated with mild disease, and importantly is not associated with the development of any human malignancy.

Adenovirus-based vectors offer several unique advantages, including tropism for both dividing and non-dividing cells, minimal pathogenic potential, ability to replicate to high titer for preparation of vector stocks, and the potential to carry large inserts. The cloning capacity of an adenovirus vector is about 8-10 kb, resulting from the deletion of certain regions of the virus genome dispensable for virus growth, e.g., E3, deletions of regions whose function is restored in trans from a packaging cell line, e.g., E1, and its complementation by 293 cells (Graham, (1977)), and deletion of E2b (Amalfitano, et al., (1998)) and E4 (Krougliak, et al.(1995); Brough, et al. (1996)) as well as the upper limit for optimal packaging which is about 105% of wild-type length.

Adenovirus DNA encapsidation occurs in a polar manner from left to right and relies on a cis-acting packaging domain located between approximately nt 200-380 (Daniell et al. (1976); Hammerskjoeld et al. (1980); Hearing et al. (1987); Robbinson et al. (1984); Tibbetts (1977)). The location of the adenovirus type 5 (Ad5) packaging domain is schematically depicted in Fig. 1A. The Ad5 packaging domain consists of at least seven redundant, albeit not functionally equivalent, elements

termed A repeats I through VII (Graeble et al. (1990); Graeble et al. (1992)).

Little is known about the identity of *trans*-acting packaging components involved in Ad packaging, but several lines of evidence support their existence in limiting concentrations in the infected cell. Most notably, cotransfection of an excess of unlinked packaging domain sequences with wild-type adenovirus genomes dramatically decreases virus yield without a significant effect on DNA replication and late transcription (Graeble, et al. (1992)). This decrease in virus growth is thought to reflect the competition of limiting, *trans*-acting packaging components from viral genomes by the unlinked packaging domain fragments, resulting in their inability to be encapsidated.

A major goal in DNA delivery systems is to create a viral vector that lacks all viral coding sequences, and only contains DNA of interest for delivery purposes plus minimal viral DNA sequences required for growth and production of the virus. To grow such a virus, a helper virus is required, but selection against contamination of the virus stock with the helper virus (wild type virus) must be imposed. The only system described to date to selectively repress packaging of an adenovirus helper virus is the excision of the packaging domain using the CRE-LOX system. This system reduces packaging of the helper virus 100- or 500-fold (Parks, et al.(1996); Hardy, et al.(1997)).

In the field of adenovirus gene therapy, the risk of RCA (replication competent adenovirus) is a major concern of developers and the FDA. RCA is the generation of wild type, infectious adenovirus via the

recombination between two different viruses within an infected cell. RCA arises primarily through homologous recombination between two viruses coinfected in a cell between overlapping homologous DNA sequences, or between virus DNA and viral DNA integrated into host chromosomes in certain complementing cell lines used to grow such a virus.

One object of the present invention is the identification of a specific control element which mediates the function of the adenovirus packaging domain. This control element operates through binding of one or more *trans*-acting proteins.

Another object of the present invention relates to the identification of a minimum packaging signal which can direct adenovirus packaging.

Yet another object of the present invention relates to adenovirus vector constructs having a selectively regulated packaging function.

Another object of the present invention relates to the use of adenovirus vectors with a regulated packaging function in a DNA delivery system.

It is another object of this invention to provide selective repression of packaging of one virus, and not another, in the context of coinfection of two viruses into cells.

It is a further object of this invention to provide a novel means to specifically repress the production of a helper virus while allowing the production of an adenovirus vector during the preparation of the virus.

Yet another object of the present invention relates to repressor-mediated control of adenovirus

particle production containing binding sites for such repressors and the use of vectors containing such binding sites for DNA delivery.

Finally, it is another object to reduce RCA in preparations of Ad virus by constructing such vectors and a helper virus with no overlap in the packaging sequences to eliminate homologous recombination.

SUMMARY OF THE INVENTION

The present invention relates to adenovirus vectors containing a minimum packaging signal for producing adenovirus virions. Of special importance is the presence of a CG dinucleotide located downstream of a TTTG sequence within each of the packaging elements. Spacing between the consensus segment 5'-TTTG-3' and the 5'-CG-3' segment located downstream is preferably between 1 and 12 nucleotides. Alternatively, it may be preferred to configure the consensus segments so that these elements appear on the same surface of the DNA helix. Most preferably, the adenovirus vector of the present invention may contain a packaging element consisting of 5'-TTTGN_8CG-3' which represents a minimal sequence necessary for adenovirus packaging. This sequence is preferably present in multiple copies. One type of minimal packaging sequence is an "A repeat", which contains a consensus sequence. Several A repeat sequences are shown in Table 1.

Another aspect of the present invention relates to novel vectors containing the minimum packaging sequences which can be selectively regulated. One such embodiment provides an adenovirus vector containing minimum packaging sequences and repressor

sites, such as COUP-TF or *lac* repressor sites. Such vectors are selectively packaged in the absence of the repressor. The repressor sites may flank the packaging sequence, may be embedded into the packaging sequence or may alternate the packaging sequence. Such vectors may contain one type of repressor site or combinations of different repressor sites.

The present invention also relates to a cellular DNA binding protein, called COUP-TF, which binds to adenovirus DNA packaging sequences. It has been found that over-expression of COUP-TF in cells infected with adenovirus specifically represses virus production; in particular, virus packaging. COUP-TF preferentially binds to certain packaging elements. Thus, adenovirus vectors of the present invention may contain one or more COUP-TF binding sites. Adenovirus vectors of the invention may contain a combination of COUP-TF binding sites and minimal packaging sequences. These elements can be used to selectively regulate packaging of such viruses.

The present invention relates to a method of regulating adenovirus packaging comprising the steps of obtaining an adenovirus vector containing a repressor binding site, propagating this vector in the absence of the repressor and repressing packaging of said vector in the presence of COUP-TF. Such a method may be carried out in one cell line. Alternatively, the propagating step may be carried out in a first cell line and the repressing step may be carried out in a second cell line. In such a system, the repressor may be endogenous to the cell line or exogenously provided at the DNA or protein level.

The present invention provides adenovirus vectors that package the virus using one or more COUP-TF binding sites or, for example, one or more A repeats. Thus, the present invention provides a selective system to control the packaging of an adenovirus vector. Optionally, the system can be designed to allow efficient packaging of one adenovirus vector while inhibiting packaging of a different vector in the same infected cell by using viruses with different packaging sites and/or COUP-TF binding sites in conjunction with COUP-TF over-expression.

Yet another aspect of the present invention provides a method of treating a patient through the administration of a heterologous gene that is expressed in the patient or a DNA fragment that is itself therapeutically active in the patient. This gene or DNA is delivered to the patient via an adenovirus vector which is prepared for administration using a regulatable adenovirus vector of the present invention.

The present invention also relates to P-complex, an activity involved in adenovirus packaging. P-complex appears to contain TATA-binding protein ("TBP") and TAF172 and is useful in production or packaging of viral particles. P-complex, interacts with the minimum packaging signal of adenovirus.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings herein.

FIG. 1 depicts the adenovirus type 5 packaging domain. (A) A schematic representation of the left end of the adenovirus type 5 genome. Nucleotide positions are indicated by numbers. The inverted terminal repeat (ITR) is represented by a gray box. Viral packaging repeats are termed A repeats I to VII (arrows). The E1A transcriptional start site is indicated by an arrow, and enhancer elements I and II are designated as E1A enhancer. (B) The packaging repeat consensus motif. Shown is an alignment of A repeats I, II, V and VI. Nucleotides comprising the bipartite consensus motif for A repeats I, II, V and VI are boxed and enlarged. The consensus motif is shown at the bottom (5'-TTTGN$_8$CG-3'). (C) Alignment of A repeats V and VI in different adenovirus subgroups: Ad 5 (subgroup C), Ad 4 (subgroup E), Ad 12 (subgroup A), Ad 3 (subgroup B), Ad 9 (subgroup D). The positions of AV and AVI are shown by horizontal lines above the sequence. Nucleotides identical between all subgroups are indicated by vertical lines.

FIG. 2 depicts the functional hierarchy among different packaging repeats. A schematic representation of left-end sequences of wild-type adenovirus is shown at the top (as per Fig. 1A). A repeats AI, AII, AV and AVI are represented by boxes of distinct shading. The mutant viruses contain a deletion between nucleotides 194 and 814, and the insertion of 6 copies each of AVI (194/814:AVI6), AII (194/814:AII6) and AI (194/814:AI6), a dimerized copy of AV, AVI and AVII (194/811:AV-AVII2) or 12 copies of AVI (194/814:AVI12). Mutant virus yields in the single infections (Yield) are expressed as fold-reduction relative to that of the wild-type virus.

The results from the coinfection experiments (Coinf.) are expressed as fold-reduction in packaged mutant DNA relative to packaged wild-type DNA. NV, virus was not viable. ND, packaged viral DNA was below the level of quantitation.

FIG. 3 depicts a cellular complex (P complex) which interacts with adenovirus packaging elements. A gel mobility shift competition experiment is presented. Radio labelled probe (AV-VII dimer) 293 nuclear extract and nonspecific competitor DNA (polydIdC) were incubated in the absence (lanes 1 and 24) or presence (lanes 2 to 23) of competitor oligonucleotides. P-complex DNA binding activity is indicated by an arrow. Increasing amounts of specific competitor oligonucleotides are indicated, and represent a 40- and 200-fold molar excess of A repeats relative to the probe. The competitors are named according to the A repeats they represent. An LS was appended when the TTTG consensus motif in the oligonucleotide was mutated. A CG was appended when the CG consensus dinucleotide was mutated.

FIG. 4 depicts P-complex and adenovirus DNA packaging. The left terminus of the adenovirus genome is schematically represented with ITR and packaging domain denoted by boxes. *Trans*-acting components binding ITR and packaging sequences are identical in the model on the left, whereas different factors interact with the respective sequences in the model on the right as indicated by circles.

FIG. 5 depicts the scheme used for P-complex purification.

FIG. 6 depicts the binding of COUP-TFI to minimal packaging domains. Gel mobility shift assays

were performed using COUP-TFI synthesized by *in vitro* translation. A hexamer of A repeat VI (lanes 1-9) and a hexamer of A repeat I (lanes 10-18) were used as radiolabelled probes. Unprogrammed reticulocyte lysate (Unprog) or increasing amounts of COUP-TFI-programmed lysate (COUP) was used in binding reactions. The addition of preimmune serum (P) or anti-COUP antiserum (α-COUP) is indicated above the lanes.

FIG. 7 depicts multimerized oligonucleotides corresponding to A repeats AI and AVI used to construct recombinant viruses. A dimeric oligonucleotide sequence is shown to simplify the schematic diagram. The potential COUP-TF binding sites in the oligonucleotides are indicated by arrows. Perfect or 4-out-of-5 nucleotide matches to the COUP-TF consensus sequence are shown as closed arrowheads; 3-out-of-5 nucleotide matches to the COUP-TF consensus site are shown as open arrowheads. Perfect, or nearly-perfect, COUP-TF binding sites with a 1 base spacing are found in multiple locations in the AVI oligonucleotide repeat, but not in the AI oligonucleotide repeat.

FIG. 8 depicts a scheme for growth of a "gutted" adenovirus gene therapy vector and the specific repression of packaging of a helper virus needed to grow the "gutted" virus. The "gutted" adenovirus lacks viral coding regions and contains the inverted terminal repeats (ITRs) required for DNA replication and a hexamer of A repeat I (for example) to direct viral DNA packaging. The remainder of the recombinant adenovirus vector is available for the insertion of large DNA segments (28 to 36 kbp). The helper virus carries all of the wild type adenovirus genome and the packaging

domain is replaced with multimerized copies (12) of A repeat VI. The helper virus is grown without COUP-TF1 overexpression to allow for the high level production of the helper virus. For the production of the "gutted" adenovirus, cells that overexpress COUP-TF1 are coinfected with the "gutted" adenovirus and the helper virus. The helper virus allows for the production of Ad early and late gene products for complementation in *trans* of the "gutted" adenovirus. However, the packaging of the DNA genome of the helper virus is specifically repressed by COUP-TF1 overexpression, while packaging of the genome of the "gutted" adenovirus is not repressed since its packaging elements do not bind COUP-TF1.

Fig. 9 depicts the specific repression of packaging of a "designer" adenovirus vector by expression of COUP-TF. (A). The growth of adenovirus USFO was measured without or with expression of COUP-TF. 293 cells were cotransfected with USFO DNA plus increasing concentrations of empty expression vector (CMX) or an expression vector for high level production of COUP-TF (CMX-COUP-TF). Virus yield (log virus yield) was measured by plaque assay on 293 cells. COUP-TF expression had a minimal effect of production of the USFO virus. (B). The growth of adenovirus USFO+AVI^{12} was measured, as described in (A). COUP-TF expression specifically repressed production of the "designer" virus USFO+AVI^{12}. The maximum level of repression of packaging of USFO+AVI^{12} by COUP-TF expression was 400-fold. (C). Western blot analysis of adenovirus late protein expression without or with COUP-TF expression. 293 cells were cotransfected with USFO DNA without or

with expression of COUP-TF. Adenovirus late protein fiber and penton were quantified by Western blot using specific antibodies. The results show COUP-TF expression has a minimal effect on adenovirus late gene expression.

FIG. 10 depicts synthetic oligonucleotides that contain different adenovirus packaging repeats designed with specific repressor binding sites that either overlap the packaging A repeats or are placed between packaging A repeats. (A) The sequence of the wild type AV-AVII oligonucleotide. A dimeric copy of this oligonucleotide efficiently directed packaging in a recombinant virus (Fig. 2). A repeats V, VI and VII are indicated and the consensus packaging repeats are encircled. (B) The AV-AVII oligonucleotide is modified (underlined nucleotides) to create a high affinity binding site for the adenovirus-induced E2F-E4-6/7 protein complex overlapping A repeats V and VI (binding site indicated by inverted arrows). (C) The AV-AVII oligonucleotide is modified (underlined nucleotides) to create a high affinity binding site for the *E. coli* lac repressor overlapping and adjacent to A repeat V (binding site indicated by inverted arrows).

Fig. 11 (A) Western blot showing lac repressor expression in 293 cells and (B) gel mobility shift assay showing lac repressor protein expressed in 293 cells binds to the AV-AVII + lac site shown in Fig. 10C.

Fig. 12 depicts the specific repression of packaging of a "designer" adenovirus vector by expression of lac repressor. The growth of adenovirus AV-VII+lac was measured without or with expression of lac repressor. 293 cells were cotransfected with AV-

° VII+lac DNA plus increasing concentrations of empty expression vector (CMX) or an expression vector for high level production of lac repressor (CMX+lac repressor). Virus yield (log virus yield) was measured by plaque assay on 293 cells. Lac repressor expression specifically repressed production of the "designer" virus AV-VII+lac. The maximum level of repression of packaging of AV-VII+lac by lac repressor expression was 20-fold.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to regulation of adenovirus packaging. Both *cis*- and *trans*-acting elements are described. These elements control adenovirus packaging, and as such, their selective use in adenovirus vectors for DNA delivery can reduce the danger of producing RCA in viral preparations and in patients.

The present invention is directed to regulatable adenovirus vectors. These new vectors have specific packaging sequences and are regulated so that production of viral particles is controlled. The vector design also increases the safety of recombinant adenovirus vectors for use as DNA transfer vehicles by reducing the potential for RCA.

The adenovirus vectors of the present invention may be derived from any known adenovirus serotype. The A repeats used as minimum packaging sequences may also be derived from any adenovirus serotype. Several example A repeats and their similarity between serotypes are illustrated in Figure 1C.

One aspect of the invention identifies that a COUP-TF binding site acts as an active site for repression of adenovirus packaging. Conversely, another aspect of the invention identifies a complex, termed P-complex which is involved in packaging. Packaging is a critical function of the adenovirus for production of viral particles. One important use for a regulated adenovirus vector is in the field of DNA delivery for therapeutic applications which uses a viral vector to deliver genes or DNAs of interest to a patient in need of such treatment.

"DNA delivery system" as this term is used herein refers to a system of delivering a DNA to a patient. Such a DNA may contain a gene encoding a protein whose expression in the patient may provide a therapeutic benefit. Such proteins may, for example, act as a treatment for a disease or condition, or may stimulate an immune response, such as a vaccine. Gene therapy is one such DNA delivery system. Alternatively, the DNA of interest may not encode a protein yet may provide a benefit to the patient. For example, a DNA may act as a antiviral agent or may transcribe into an RNA which may act as an antisense therapeutic or antiviral agent.

The present invention also relates to the identification of a minimum adenovirus packaging signal. A minimal packaging sequence of 5'-TTTGN$_8$CG-3' has been identified. Although eight nucleotides are preferred to separate the left portion of the packaging consensus element (i.e., 5'-TTTG-3') from the right portion (i.e., 5'-CG-3'), this spacing may vary 1 to 12 nucleotides. Alternatively, it may be preferred to configure the

consensus segments so that the segments appear on the same surface of the DNA helix. The packaging element may be inserted into the left or right end of the adenovirus vector, preferably within 600 nucleotides from either end. More preferably, this minimal sequence is present at the left end of the genome and is present in multiple copies. Another consensus sequence comprises 5'-ATTTGN$_8$CG-3' and provides a strong packaging signal in adenovirus vectors. Two copies of this minimal packaging sequence are sufficient to ensure packaging. More than two copies enhance virus packaging. However, any number of this sequence can be inserted into the virus to ensure particle production. "Multimerized" as this term is used in the instant application refers to multiple copies of an element (i.e. packaging or repressing). These elements may be present in single units or in multimers, which preferably means 2-36 repeats and more preferably 2-12 units or elements. One form of the minimal packaging element is an "A repeat", which is derived from adenovirus. Representative A repeats are set forth below in Table 1:

TABLE 1

AI:	5'-TTTGGGCGTAACCG-3'
AII:	5'-TTTGGCCATTTTCG-3'
AIII:	5'-TCTGAATAATTTTG-3'
AIV:	5'-TTTGTGTTACTCAT-3'
AV:	5'-TTTGTCTAGGGCCG-3'
AVI:	5'-TTTGACCGTTTACG-3'
AVII:	5'-TTTACGTGGAGACT-3'

Unique adenovirus vectors that contain minimal packaging domains have been developed consisting of

multimerized oligonucleotide sequences in place of the normal packaging domain. Additionally, these new adenovirus vectors may contain deletions of viral DNA sequences from the left end of the genome which allow for augmented insertion of foreign DNA sequences in the context of DNA delivery vectors. Up to 400 nucleotides can be deleted from the left end of the genome and be replaced with the minimum packaging sequences defined herein to produce a vector with an increased capacity to carry foreign DNA. Further, the use of different packaging oligonucleotide repeats in different individual viral vectors allows for the selective repression of packaging of one adenovirus vector, but not another adenovirus vector, in cells coinfected with both viruses. The latter scenario is important in the design of a vector capable of selective packaging for use in DNA delivery systems, and the repression of packaging of a helper virus needed to grow the adenovirus vector.

The vectors of the present invention are useful in DNA delivery systems to help curb the production of replication competent adenovirus (RCA), a virus that is dangerous and potentially toxic to a patient receiving it during patient administration. This is due to the fact that two distinct viruses can be made with entirely distinct, and non-overlapping packaging domains. For example, a virus (eg. gutted gene therapy virus 1) may contain a hexamer of A repeat I in direct orientation, while a helper virus (virus #2) may contain a dimer of A repeats V, VI and VII or a multimer of AVI in an inverted orientation. Thus, both viruses carry functional packaging domains, but overlap

homologous recombination is greatly minimized since different packaging sequences and DNA orientations are used. A target for homologous recombination does not exist in the packaging domain. In such coinfection conditions, the use of different packaging domains in the two viruses greatly minimizes the possibility of recombination between the two viruses to generate RCA.

In one embodiment of the present invention, one or two copies of a DNA segment containing packaging A repeats V, VI and VII direct packaging. A single copy of the segment functions for packaging. This type of packaging sequence contains a series of different repeats and is referred to as a natural packaging domain. The second type of packaging sequence contains a single type of A repeat which when multimerized functions efficiently for packaging. This segment is referred to as a synthetic packaging element. Vectors of the present invention may contain a combination of natural and synthetic packaging elements.

The present invention approach to DNA delivery vector design preferably uses a "gutted" adenovirus vector whereby most or all of the viral genes are removed. There are two advantages with "gutted" vector approach. First, little or no viral proteins are produced following infection that normally elicit an immune response. Second, such a virus is capable of carrying very large gene inserts for gene therapy applications. For example, the dystrophin gene for treatment of muscular dystrophy is 14,000 bp in length necessitating a vector with very large insert capacity. Also, the Factor VIII gene for treatment of hemophilia A is greater than 7000 bp. Additionally, it may be

preferable to use tissue-specific regulatory sequences to produce tissue-specific expression of a gene. This requires increasing the insert capacity in a vector, because many tissue-specific promoters contain several thousand base pairs.

Many genes and/or DNA segments may be carried by adenoviral vectors. Examples of such genes include; interleukin-2 (Haddada, et al. (1993)) p53 (Harris, et al. (1996)); α1-antitrypsin (Jaffe, et al.(1992), cystis fibrosis transmembrane conductance regulator (CFTR) (Rosenfeld et al., (1992)), and clotting factor VIII (Connelly, et al. (1995)).

The recombinant adenovirus of the present invention is preferably a "gutted vector" and contains adenovirus sequences at the left and right termini required for DNA replication and two or more copies of the minimal packaging sequence to direct viable DNA packaging. The remainder of the recombinant adenovirus vector is available for insertion of large DNA segments (up to 36,000 base pairs). A helper adenovirus is needed to grow such a "gutted" vector in order to produce all of the viral proteins that are missing in the "gutted vector".

In DNA delivery systems, there are circumstances in which it is desirable to prevent production of a viral particle. In particular, helper virus, a virus necessary for replication of the viral construct, is highly undesirable in the preparation for patient administration. According to one embodiment of the present invention, a helper virus is designed to contain a COUP-TF binding site and is first allowed to grow productively in the absence of COUP-TF, then is

blocked from being packaged by the presence of COUP-TF. In this embodiment, the viral growth is carried out in a cell line which does not express COUP-TF and the packaging is blocked by the addition of COUP-TF protein. In a second embodiment, the viral growth is carried out in a cell line lacking COUP-TF (Qiu, et al. (1997)) and the packaging repression step is accomplished by transfer of the virus into cells expressing COUP-TF. In this way, helper virus can be used to propagate the adenovirus vector yet not be present in the final viral preparation.

Another important aspect of the present invention relates to gene therapy vectors that use adenovirus minimal packaging sequence, 5'- $TTTGN_8CG$ -3'. (See Provisional patent application no. 60/081,867, incorporated herein by reference).

One preferred adenovirus vector design of the present invention utilizes a packaging/repressor system. In this embodiment, adenovirus vectors are constructed with alternating oligonucleotides containing the minimal packaging sequence and binding sites for a repressor. For example, a *lac* repressor site can be inserted between packaging sequences. The *lac* repressor is a high affinity binding repressor not found in eukaryotic cells. Another example of such a system embeds one or more repressor sites within a packaging domain. Yet another example of a packaging/repressor system flanks a packaging domain with surrounding repressor binding sites. This system may have one or a series of repressor binding sites to the left of a minimal packaging domain and another set of repressor binding sites to the right of a packaging domain. Thus, a virus

which contains minimal packaging sequence and repressor binding sites such as, for example, *lac* repressor sites, can be grown in cells not expressing the repressor, and then packaging can be selectively repressed in cells expressing high levels of the repressor.

The present invention also provides vectors containing a packaging sequence in combination with the COUP-TF repressor binding sites whose packaging capability can be selectively controlled. For example, such vectors may have a packaging sequence containing a dimer of A repeats V, VI. These packaging domains may also contain a COUP-TF repressor site as well as signals sufficient to allow efficient packaging. Such vectors allow packaging in the absence of COUP-TF repressor, but inhibit packaging in the presence of COUP-TF.

For the production of the recombinant adenovirus of the present invention, cells that overexpress COUP-TF1 can be infected with the therapeutic adenovirus vector containing one type of packaging element (for example, multiple copies of A repeat I) and the helper Ad containing a different type of packaging element (for example, multimerized copies of A repeat VI). The packaging of the helper virus will be specifically suppressed by COUP-TF1 overexpression, while packaging of the genome of the adenovirus gene therapy vector will not be repressed. A conditional system for repression of packaging is designed into the vector so that a helper virus can be grown to high levels under non-repression conditions, and then specific repression of the helper virus packaging accomplished when used to complement growth of the therapeutic virus vector.

In another vector embodiment of the present invention, *lac* repressor binding sites are embedded within a minimal packaging domain. For example, a packaging domain may be engineered to contain a *lac* repressor binding site embedded within the A repeat V, VI and VII packaging domain. The virus can then grow in the absence of *lac* repressor expression while repression of packaging (e.g. a helper virus) is observed with high level *lac* repressor expression. The virus can then grow in the absence of lac repressor expression while packaging is repressed when *lac* is expressed.

In yet another vector embodiment of the present invention, E2F transcription factor binding sites are embedded within a minimal packaging domain. The idea is the same as directly above, i.e. a high affinity binding site for a DNA binding protein is embedded within a minimal packaging domain with the ability to selectively "activate" the repressor. In this embodiment, the cellular transcription factor (E2F) and an adenovirus protein (E4-6/7) which induces the cooperative and stable binding of E2F to an inverted binding site provide the packaging/repressor system of this vector. A high affinity E2F inverted binding site is inserted within a minimal packaging domain containing, for example, A repeats V, VI and VII. In the absence of 6/7 protein expression (this mutant virus is completely viable), E2F binding to the packaging region is weak and thus repression is weak. In the presence of the E4-6/7 protein, E2F binding is stable and with high affinity. Thus, binding of the bona-fide packaging factor is repressed and packaging of the virus is blocked.

Alternatively, binding sites for other repressors, such as, for example, lambda repressor or Tet repressor, can be employed in the design of adenovirus vectors of the present invention. Other potential repressor sites can be employed and will be readily known to the skilled artisan.

Multimers of different A repeats are able to direct packaging of viral DNA but at different efficiencies (Schmid and Hearing, 1998). Any of the A repeats may serve as a minimal packaging sequence. Preferably these A repeats are used as multimers in a packaging element. A dimer of A repeats V-VII and a hexamer of A repeat I, most preferably as a multimer, serve as the most efficient packaging domains *in vivo*. A hexamer of A repeat II can also be used in the present invention, having a moderate activity. A hexamer of A repeat VI is also a packaging element, albeit a weak element. A repeat VI, when utilized as a multimer, preferably a 12-mer, efficiently directs packaging.

One embodiment of the present invention relates to vector constructs containing multimers of the A repeat VI packaging signal which is a high affinity binding site for COUP-TF binding. Such a vector construction can be regulated through selective expression of COUP-TF.

In light of the fact that COUP-TF binds to adenovirus packaging sequences, the effect of overexpression of COUP-TF on adenovirus infection was tested. Overexpression of COUP-TF resulted in a 10,000-fold decrease in the production of infectious adenovirus. This effect was, at least in part, due to repression of the adenovirus major late promoter ("MLP")

which directs the synthesis of adenoviral late mRNAs and thus viral late proteins. COUP-TF binds to a specific DNA sequence in the MLP that overlaps the binding site for the activating transcription factor called USF (Sawadogo and Roeder, 1985). COUP-TF is a known repressor of eukaryotic transcription promoter activity (Cooner et al., 1992; Tsai and Tsai, 1997).

In another embodiment of the invention, P-complex was found to interact with cellular complexes in the viral packaging machinery (Schmid and Hearing, 1998). A direct correlation is seen between the binding affinity of P-complex for different A repeats *in vitro* and the ability of the respective fragments to support DNA packaging *in vivo*. The TTTG, but not the CG, packaging consensus half site is critical for P-complex interaction. In addition the P-complex binds to core replication sequences in the inverted terminal repeat (ITR). The cellular P-complex activity, by virtue of its ability to interact with both packaging and core replication sequences, constitutes a *trans*-acting link between viral DNA replication and encapsidation. The binding of a cellular transcription factor, COUP-TF, to minimal segments of the viral packaging domain was also detected. Its binding affinity does not correlate with viral DNA packaging *in vivo*, but rather repression thereof.

Cellular P-complex is a bona-fide adenovirus packaging component. This complex appears to contain a TATA binding protein (TBP) and a second protein called TAF172 (Timmers et al. 1992, Taggart et al. 1992). P-complex binding is inhibited by ATP and magnesium. Complex formation is observed on all minimal packaging

domains that exhibit functional activity *in vivo*. The affinity of the P complex for the different multimeric A repeats *in vitro* correlates well with the ability of the respective cis-acting sequences to support viral DNA packaging *in vivo*. Specifically, AI and AV-VII constitute strong P complex binding sites and they confer maximal packaging activity *in vivo*. The most preferred P-complex binding sites comprise a hexamer of AI and a dimer of AV, AVI and AVII. On the other hand, AVI is noted as a weak binding site for P complex *in vitro*, and it serves as a particularly weak packaging domain *in vivo*. As discussed above, the Ad packaging consensus motif is a bipartite sequence with a conserved AT-rich and a GC-rich half site (5'TTTGN$_8$CG-3') (Schmid, et al. (1997)).

The identification of the DNA binding activity of P complex as containing TBP-TAF172 has important implications for the development of "designer" adenovirus vectors for repression of packaging. For example, using the viruses depicted in Fig. 8, a gutted gene therapy vector may be generated that binds P complex/TBP-TAF172 poorly using mutations in the AT-rich binding site that reduce TBP binding to DNA in the helper virus packaging sequences. Additionally, so-called "altered-specificity" TBP mutants may be used in the present invention (Strubin and Struhl, 1992). Such mutations produce TBP protein having altered specificity for binding to certain DNAs. That is, the altered-specificity TBP mutant binds to a TATA box sequence with a nucleotide change (TATA to TGTA), whereas the normal wild type TBP in the cell is unable to bind such a TGTA site efficiently. Thus, adenovirus vectors with altered

specificity P complex/TBP-TAF172 binding sites may be constructed to conditionally repress packaging of a helper virus. The helper virus contains the altered specificity TGTA binding site in place of the AT-rich part of the A repeat; the virus can be successfully propagated when altered-specificity TBP is provided in cells, and packaging of the helper virus repressed when grown in cells lacking the altered-specificity TBP. Other manipulations of the P complex/TBP-TAF172 binding site and/or manipulations of the DNA binding proteins can be made by the skilled artisan toward the same goal.

Our working model, shown in Figure 4, is based on the data from protein binding studies presented in the Examples. A coordinate interaction of packaging factors with viral A repeats is shown. Three copies of A repeats are preferred for efficient DNA encapsidation (Graeble et al. (1990); Graeble et al. (1992)), which likely reflects the need for the presence of multiple protein binding sites. Either the same or a different *trans*-acting component may bind the left-end 13 nt of the adenovirus genome. Physical association between the components bound to ITR and packaging sequences results in the formation of a nucleoprotein complex within the viral left end, marking the respective molecule as a bona-fide packaging substrate. This complex corresponds to the P-complex detected in our gel mobility shift assays since it exhibits binding specificity for both packaging and ITR sequences. The AT-rich packaging consensus half site is implicated in the initial recognition of A- repeats by packaging factors. Perhaps the CG-rich half site and proteins bound to it are involved in secondary events like capsid recognition or

insertion of the viral DNA into the capsid. It is noteworthy that the 8 bp spacing, or one helical turn of the DNA, which separates the AT-rich and the CG-rich consensus half site is important for DNA encapsidation *in vivo*. This may reflect the need for a physical interaction between components of the P-complex and CG-bound unidentified components, to allow for the timing and/or coordination of successive steps in adenovirus DNA packaging.

While the invention is described above in relation to certain specific embodiments, it will be understood that many variations are possible, and the alternative materials and reagents can be used without departing from the invention. In some cases such variations and substitutions may require some experimentation, but will only involve routine testing.

The foregoing description of the specific embodiments will fully reveal the general nature of the invention and others can, by applying current knowledge, readily modify and/or adapt for various applications or such specific embodiments without departing from the generic concept, and therefore such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments.

All articles, patents or other references cited or referred to herein are hereby incorporated herein in toto by reference.

EXAMPLES

MATERIALS AND METHODS

Virus constructions. Ad5 dl309, the parent for all the viruses described in this report, is a phenotypically wild type virus that contains a unique XbaI cleavage site at 3.8 map units (Jones, et al. (1979)). Plasmid pE1A-194/814 contains the left end Ad5 XbaI fragment (nt 1-1339) with a deletion between nt 194 and 814 and a unique XhoI restriction site at the junction of the deletion. A head-to-tail hexamer of an oligonucleotide containing A repeat VI (5'-TCGACCGCGGGGACTTTGACC-3': 5'-TCGAGGTCAAAGTCCCCGCGG-3') was cloned into the 194/814 deletion. Similarly, head-to-tail hexamers of oligonucleotides containing A repeat I (5'-TCGAGTTGTAGTAAATTTGGG-3': 5'-TCGACCCAAATTTACTACAAC-3') or A repeat II (5'-TCGACCGAGTAAGATTTGGCC-3': 5'-TCGAGGCCAAATCTTACTCGG-3') were cloned into the pE1A-194/814 background. pBR-194/814 and pBR-53/814 have sequences between nt 194 and 814 and nt 53 and 814 deleted. A monomer and dimer of viral sequences is located between nt 334 and 385 which contain AV, AVI, and AVII was cloned into the 194/814 deletion. A dimer of the nt 334 to 385 fragment as well as 12 head-to-tail copies of an oligonucleotide containing AVI (5'-TCGACCGCGGGGACTTTGACC-3':5'-TCGAGGTCAAAGTCCCCGCGG-3') were cloned into the 53/814 deletion in either orientation. All mutations were verified by nucleotide sequence analysis.

The recombinant plasmids were subsequently rebuilt into intact viruses by the method of Stow (1981). Viruses were amplified and titered on 293 cells. Mutant viruses were screened by restriction analysis of viral DNA obtained from infected 293 cells by the Hirt procedure (Hirt (1967)), and all insertions were verified by nucleotide sequence analysis of viral DNA using PCR-based sequencing.

Cultured cells and infections. Virus stocks were generated by three freeze-thaw cycles of infected cell lysates and titered by plaque assays on 293 cells. Virus infections were performed at a multiplicity of infection (MOI) of 3 PFU per cell for 1 h at 37°C. Cells were then washed twice with tris-buffered saline solution and overlayed with fresh medium.

Determination of virus yield and packaging efficiency. Both assays were performed as described previously (Schmid, et al. (1997)). For the determination of virus yield in a single infection, infected cell lysates were prepared 48 h post-infection and the amount of infectious virus was determined by plaque assays on 293 cells. Packaging efficiency of the mutant viruses was tested in a coinfection of 293 cells with both mutant and wild-type dl309 virus. Forty-eight hours post-infection, one half of the cells was used to isolate total nuclear DNA, the other half was used for the preparation of viral DNA from purified virions. Both DNA preparations were digested with XbaI to distinguish between mutant and wild-type DNA and quantitated by Southern blot hybridization using pE1A-WT, ^{32}P-labeled by the random primer method (Feinberg, et al. (1983)), as a probe. The relative intensities of the bands in

autoradiograms were determined by densitometric scanning. Quantitation of the data was performed by using the public domain NIH Image program (written by Wayne Rasband at the National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS,5285 Port Royal Rd., Springfield, VA 22161, part no. PB93-504868). The data presented for virus yield in the single infections and the data for packaging efficiency based on coinfection experiments represent the averages of three to five independent experiments.

The data presented for virus yield in the single infections and the data for packaging efficiency based on coinfection experiments represent the averages of at least three independent experiments.

Extract preparation and gel mobility shift assays. Nuclear extracts were prepared by the method of Dignam and Roeder (1983), and dialyzed overnight against 20 mM N-2hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.5), 100 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (DB-100). The dialysate was cleared by centrifugation at 25,000 x g for 20 minutes. Two to five grams of nuclear extract was incubated with 0.5µg of polydeoxyinosinic-deoxycytidylic acid (poly dIdC) and 20,000 cpm of ^{32}P-labeled probe DNA (2.5 to 5 fmol of DNA) per *in vitro* binding reaction. The binding reaction was carried out in a total volume of 20 µg for 1-2 hr at room temperature in 40 mM HEPES pH 7.5, 70 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonylfluoride, 10 µg/ml BSA and 4% Ficoll. The complexes were resolved electrophoretically

at 10 V/cm on a 3.5% 30:1 (acrylamide: bisacrylamide) polyacrylamide gel in 0.5x TBE (25 mM Tris pH 8.3, 25 mM boric acid, 0.5 mM EDTA) at 4°C. For gel mobility shift assays performed with *in vitro* translated COUP-TFI protein, 0.25-1.5 µl of rabbit reticulocyte extract programmed with *in vitro* synthesized RNA transcript encoding COUP-TFI was assayed using the binding conditions described above. *In vitro* transcription and translation was performed as recommended by the manufacturer (Promega). For gel mobility supershift experiments, 0.5 µl of a rabbit polyclonal anti-COUP antiserum (a gift from Dr. Alonzo D. Garcia) was added after a one hour binding reaction, and incubation was then continued for an additional 30 minutes.

Plasmids, probes and competitor fragments. Head-to-tail hexamers of A repeats I and VI, individually, and a dimer of A repeats V-VII were cloned into pUC9. The sequence of a monomer of A repeat I is: 5'-TCGAGTTGTAGTAAATTTGGG-3': 5'TCGACCCAAATTTACTACAAC-3', a monomer of A repeat VI is: 5'--TCGACCGCGGGGACTTTGACC-3': 5'-TCGAGGTCAAAGTCCCCGCGG-3'. A monomer of AV-VII is: 5'-TCGACCGCGTAATATTTGTCTAGGGCCGCGGGGACTTTGACCGTTTACGTGGAGACT

CC-3':5'

TCGAGGAGTCTCCACGTAAACGGTCAAAGTCCCCGCGGCCCTAGACAAATATTACGCGG-3'. The fragments were liberated from the vector by digestion with EcoRI and HindIII, gel purified and ^{32}P-end-labeled with Klenow DNA polymerase and (α-^{32}P)dATP. For the preparation of ITR 1-13 probe, a monomeric oligonucleotide representing the left end 13

nt flanked by Xho/Sal linkers (5'-TCGACATCATCAATAATC-3':5'TCGAGATTATTGATGATG was end-labeled in the same way using (α-^{32}P)dCTP.

For the preparation of competitor fragments containing packaging repeats, monomeric oligonucleotides were multimerized using T4 DNA ligase. Selection for head-to-tail multimers was achieved by subsequent digestion using SalI and XhoI, followed by phenol/chloroform extraction and ethanol precipitation. In addition to multimers prepared from the oligonucleotides representing packaging elements I, VI and V-VII described above, A repeat II (5'-TCGACCGAGTAAGATTTGGCC-3':5'-TCGAGGCCAAATCTTACTCGG-3') and A repeat V (5'-TCGACCGCGTAATATTTGTCC-3': 5'-TCGAGGACAAATATTACGCGG-3') were used as multimeric competitors. Packaging repeat competitor fragments designated LS have the underlined nucleotides shown above in AI, AII, AV, AVI, AV-VI mutated into the sequence 5'GTGCAG-3' (only the upper strand is indicated). The italicized CG dinucleotide in the AV competitor was replaced by an AT in the competitor fragment designated CG. The competitor oligonucleotide representing ITR sequences 1-13 was used in monomeric form and was identical to the one used for probe preparation. The monomeric ITR 10-22 competitor oligonucleotide contains sequences between Ad nt 10-22 flanked by XhoI/SalI linkers. Quantitation of oligonucleotide competitors was performed spectrophotometrically. The amount of specific competitor DNA added per binding reaction is indicated in the text as -fold molar excess of binding sites present in the competitor relative to binding sites

present in the probe. This definition, however, is based on the assumption that one binding site (located between nt 1-13) is present in monomeric ITR fragments and that six binding sites are present in hexameric packaging repeat fragments.

Western blot analysis. Proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose, and probed with different antibodies (rabbit polyclonal anti-COUP, anti-fiber and anti-penton antisers, monoclonal antibody M45). Proteins were visualized using a secondary horseradish peroxidase-conjugated antibody and chemiluminescence as recommended by the manufacturer (Amersham).

EXAMPLE 1

Minimal adenovirus packaging domains. Adenovirus packaging elements are functionally redundant, but in spite of this redundancy, different elements are not functionally equivalent with respect to each other. Elements I, II, V and VI constitute the most functionally dominant A repeats (Graeble et al. (1990); Graeble et al. (1992); Schmidt et al. (1997)). The selection of revertant adenoviruses from a packaging deficient parent virus has been defined A repeat VI as an independent cis-acting unit (Schmid, et al. (1997)). A hexamer of A repeat VI in place of the packaging domain yields a viable virus, although the mutant is reduced >100-fold in growth compared to wild-type. Such a mutant is under strong evolutionary pressure for the amplification of packaging elements since revertants with significantly improved growth were found to evolve by amplification of preexisting copies of A repeat VI.

In contrast, a fragment containing A repeats V-VII functions efficiently to direct packaging and these A repeats did not amplify upon virus propagation (Fig. 2; Schmid, et al. (1997)). Sequences flanking the packaging domain are identical in both of these mutant viruses (a deletion of sequences between nt 194 and 814). This raises the question of whether there is a hierarchy of importance among the four most- dominant A repeats with A repeat VI as a functionally less dominant element, or alternatively, whether a combination of different elements supports packaging better than only one type of A repeat.

To begin to address the first possibility, viral mutants were constructed that contain multimers of individual A repeats inserted into a 194/814 deletion background (Fig 2) (Schmid and Hearing, (1998)). The packaging domain was replaced by a hexamer in the forward orientation of AVI, AII and AI, respectively. The parent virus was nonviable (described Schmid, et al. (1997)), and lacking any functional packaging elements. Insertion of a hexamer of AVI, AII and AI into the 194/814 deletion background rescued virus viability, albeit to different extents. A multimer of A repeat VI in place of the packaging domain resulted in a virus that exhibited a more than 100-fold reduction in growth in a single infection relative to wild-type virus. DNA packaging in a coinfection with wild-type virus was nondetectable. A virus with 12 copies or more of AVI packaged at wild type efficiency. A hexamer of repeat I supported viral growth in a single infection and DNA packaging in a coinfection better than A repeat II, with a reduction in growth of 4-fold versus 20-fold in the

single infection and in packaging efficiency of 2-fold versus 5-fold in the coinfection, respectively. These results suggest that there is a hierarchy of functional importance within the group of most efficient packaging elements, with element VI as the weakest element followed by A repeat II and finally A repeat I as the functionally most dominant A repeat.

EXAMPLE 2

P complex purification scheme (Figure 5). HeLa cell pellets were obtained from the National Cell Culture Center (Minneapolis, MN). All procedures were performed at 4°C. Nuclear extract was prepared by the method of Dignam et al. (1983), dialyzed into buffer DB-100 (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 20% glycerol, 0.1 mM EDTA, 0.5 mM PMSF, 0.25 mM benzamidine, 1.0 mM DTT), and the dialysate centrifuged at 25,000 x g for 20 minutes. Buffer DB is the same buffer but lacks NaCl. Nuclear extract was applied to a heparin-agarose column (10 mg protein/1 ml heparin-agarose) equilibrated in DB-100, the column was washed with DB-100, and bound proteins were eluted with a linear NaCl gradient (0.1 M-0.6 M) in DB. Fractions containing P complex activity were identified using a gel mobility shift assay with a DNA probe consisting of a dimer of A repeats V-VII (as per Fig. 3). The P complex peak eluted at 0.42 M NaCl. The NaCl concentration was diluted to 0.05 M using DB, and the P complex pool was applied to a phosphocellulose P11 column (8 mg protein/1 ml P11) equilibrated in DB-0.05, the column was washed with DB-0.05, and bound proteins were eluted with a linear NaCl gradient (0.05 M-0.6 M) in DB. The peak of P complex activity eluted

at 0.13 M NaCl. The P complex pool was diluted to 0.1 M NaCl using DB, and applied to an SP-Sepharose column (8 mg protein/1 ml SP-Sepharose) equilibrated in DB-100. The column was washed with DB-100, and bound proteins eluted with a linear NaCl gradient (0.1 M-0.6 M) in DB. The peak of P complex activity eluted at 0.20 M NaCl. The P complex pool was diluted to 0.1 M NaCl using DB and protease inhibitors aprotinin and leupeptin were added to 1 μg/ml to all buffers from this point on. The P complex pool was applied to a Q-Sepharose column (8 mg protein/1 ml Q-Sepharose) equilibrated in DB-100. The column was washed with DB-100, and bound proteins eluted with a linear NaCl gradient (0.1 M-0.6 M) in DB. The peak of P complex activity eluted at 0.28 M NaCl. The P complex pool was diluted to 0.1 M NaCl using DB, and $NaPO_4$ was added to 10 mM. The P complex pool was applied to a hydroxy-apatite column (5 mg/protein/1 ml hydroxy-apatite) equilibrated in DB-100+ 10 mM $NaPO_4$. The column was washed with DB-100 + $NaPO_4$, and bound proteins eluted with a linear $NaPO_4$ gradient (10 mM - 250 mM) in DB-100. P complex activity was pooled with a final purification of ~1000-fold.

EXAMPLE 3

A cellular complex (P-complex) interacts with adenovirus packaging elements. Minimal packaging domains defined *in vivo* were used as probes for gel mobility shift assays for the detection of *trans*-acting packaging components. Since such components could be viral and/or cellular in origin, we initially carried out binding studies with both uninfected and Ad-infected 293 cell nuclear extracts. Infections were performed

using either wild-type Ad dl309 or a temperature-sensitive virus, ts19, defective for virus assembly when grown at the restrictive temperature (Williams, et al. (1971)). Extracts from ts19-infected cells were tested in view of the fact that packaging factors may be encapsidated with wild-type adenovirus and consequently not present in nuclear extracts used for *in vitro* binding studies. At no point did we detect any difference between complex formation using nuclear extracts from infected or uninfected cells, and therefore, all experiments presented below were performed with extracts from uninfected cells.

A fragment containing a dimer of A repeats V-VII confers wild-type packaging abilities *in vivo* to a mutant virus which lacks the packaging domain (Schmid, et al. (1997)). Figure 3 shows the results from a gel mobility shift assay in which this fragment was used as a probe and incubated with uninfected 293 cell nuclear extract for the detection of interacting proteins. In lanes 1 and 24 (+), no specific competitor was added, whereas a 40- and 200-fold molar excess of competitor oligonucleotides were added to the binding reactions resolved in lanes 2 to 23. The specific competitor fragments are indicated above the autoradiography and represent different multimeric A repeats, either in the wild-type or mutated configuration (see Materials and Methods for names and sequences). A slow migrating complex, termed the P-complex (indicated by an arrow) was formed on the AV-VII probe (lanes 1 and 24), which disappeared upon self-competition (lanes 2 and 3), but not when the TTTG half-site of the packaging element

consensus motif was mutated in A repeats V and VI of the competitor oligonucleotide (lanes 4 and 5).

In a similar fashion, the addition of fragments representing AVI (lanes 6 and 7), AV (lanes 10 and 11), AI (lanes 16 and 17) and AII (lanes 20 and 21) resulted in competition for P-complex formation, but not when the consensus TTTG half-sites were mutated (lanes 7, 8, 12, 13, 18, 19, 22 and 23). The efficiency of individual A repeats to compete for P-complex binding in a gel shift assay can be rated, with AV-VII and AI as the best competitors, followed by AII as an intermediate competitor and AVI as the weakest competitor. This correlates with the ability of the respective fragments to function individually as packaging domains *in vivo* (Fig. 2). Mutating the CG dinucleotide within the competitor oligonucleotide did not affect complex formation as exemplified by efficient competition observed with the AVCG competitor oligonucleotide (lanes 14 and 15) indicating that the CG consensus half site is not critically involved in P-complex binding. Other competitor oligonucleotides representing different A repeats with mutations in the CG dinucleotide were also tested, and identical results were obtained. P-complex formation was also observed using HeLa cell nuclear extract.

In summary, a cellular binding activity, termed P-complex, interacts specifically with various packaging elements in a gel mobility shift assay, in perfect correlation with data obtained *in vivo* with mutant viruses containing minimal packaging domains. Integrity of the AT-rich, but not the CG-rich, part of

the packaging consensus motif is critical for this interaction.

EXAMPLE 4

P complex interacts with viral core origin sequences. P complex binding activity was bound to bind to sequences derived from the left terminus of the adenovirus genome (Schmid and Hearing,1998). Using gel mobility shift assays, the binding of P complex to A repeat sequences (AI hexamer probe or AV to VII dimer probe) was efficiently competed by an oligonucleotide containing left en ITR sequences from nucleotides 1 to 13, but not by an oligonucleotide containing ITR sequences from nucleotide 10 to 22. Similarly, P complex bound efficiently to a DNA probe containing ITR sequences from nucleotides 1 to 13, and this binding was efficiently competed by wild type A repeat oligonucleotide competitors, but not by A repeats with mutation in the TTTG consensus motif. The data show that P complex not only binds to packaging A repeats, but also to the very terminus of the adenovirus genome (nucleotides 1 to 13). As depicted in Fig. 4, the binding of P complex to the packaging domain and left terminus of the adenovirus genome followed by P complex protein-protein interaction may result in looping of the intervening DNA sequences. The competition experiments are consistent with one or two possibilities for P complex binding activity. First (Fig. 4 LEFT), P complex may contain one DNA binding activity that recognizes both packaging A repeats as well as the left terminus of the adenovirus genome (which is AT-rich but does not have a consensus A repeat sequence). Second (Fig. 4

RIGHT), P complex may consist of two distinct but interacting activities whereby one DNA binding activity binds the consensus A repeat sequence and the second DNA binding activity binds to the AT-rich left terminus of the adenovirus genome.

EXAMPLE 5

COUP-TF interacts with adenovirus packaging elements. Database searches revealed that the AVI probe contains highly conserved dimeric consensus binding sites for a cellular transcription factor, chicken ovalbumin upstream promoter transcription factor (COUP-TF; Cooney et al. (1992)). COUP-TF binds to the consensus sequence 5'-GGTCA-3' when situated as a direct or inverted repeat, with a preferred spacing of 1 base pair, and represented as perfect or imperfect versions of the consensus binding site. These binding sites overlap A repeat VI (5'-GGACTTTGACC-3'; the COUP-TF inverted repeat is underlined, and AVI is in bold), only the upper strand is indicated with the COUP half sites underlined and AVI indicated in bold case. Other A repeats contain similar sequence motifs, albeit with less resemblance to the dimeric COUP consensus.

In view of the conserved COUP-TF binding motif contained within AVI, we asked whether the multimeric protein-DNA complexes formed on the AVI probe in particular, but also complexes formed on other A repeats, might contain COUP-TF (Schmid and Hearing, 1998). Heparin agarose fractions were subjected to Western blot analysis using a polyclonal COUP-TF antiserum. A band of approximately 45Kd molecular size was detected in fractions 24 to 31, which represents a

low-molecular form of COUP-TF. The presence of COUP-TF protein in fractions 24 to 31 correlates with the presence of a packaging repeat binding activity which exhibits striking affinity for A repeat VI.

To test COUP-TF binding to A repeats I and VI directly, we performed gel mobility shift assays using *in vitro* transcribed and translated COUP-TFI with hexameric AVI and AI probes (Fig. 6). COUP-TFI strongly bound to the AVI probe (lanes 4 to 7), and weakly to the AI probe (lanes 13 to 16). Addition of polyclonal COUP-TF antiserum (lanes 9 and 18), but not preimmune serum (lanes 8 and 17), resulted in the formation of a supershift in each case. The formation of weak complexes on both probes by the addition of unprogrammed reticulocyte lysate alone (lanes 1 and 10) was observed. No supershifts, however, were formed upon the addition of either preimmune serum (lanes 2 and 11) or COUP-TF antiserum (lanes 3 and 12) suggesting that COUP-TF is not contained within these complexes. Probes representing AII and AV-VII bound COUP-TF with similar affinity to the AI probe. COUP-TF, when synthesized *in vitro*, displays sequence-specific binding affinity for all minimal packaging domains. COUP-TFI exhibits lowest binding affinity for AI and highest binding affinity for AVI, opposite to the ability of the respective elements to serve as minimal packaging domains *in vivo*.

Sequence-specific binding of COUP-TF to viral packaging elements provide another level of regulation of adenovirus packaging (Schmid and Hearing, 1998). COUP-TF binds to A repeats when synthesized *in vitro* (Fig. 6) or when expressed using baculovirus. Highest affinity was observed for A repeat VI multimers. Also,

heparin agarose chromatography correlated the peak of binding activity interacting with A repeat VI multimers with peak levels of COUP protein. Further, gel mobility supershift experiments using minimal packaging domains as probes showed the presence of a COUP-related binding activity in uninfected nuclear 293 and Hela cell extracts.

EXAMPLE 6

To generate a "designer" adenovirus vector where COUP-TF specifically represses adenovirus packaging, a virus referred to as helper virus in Fig. 8 containing the USF-0 mutations in the MLP was generated. The salient features of the vector are: mutations of the COUP-TF binding site in the MLP (USF-0) so MLP activity is not repressed by COUP-TF overexpression *in vivo*, and 12 copies of A repeat VI in place of the normal adenovirus type 5 packaging domain (nt. 194-452). This new "designer" helper virus vector is termed USF-0 + AVI^{12}. A repeat VI is a high affinity COUP-TF binding site. USF-0 DNA or USF-0 + AVI^{12} DNA was cotransfected with a COUP-TF high level expression vector (CMX-COUP-TF) or with a control vector (CMX) into human 293 cells. Two days later, production of infectious virus was assayed. The results (Fig. 9) showed that COUP-TF expression specifically repressed production of the "designer" virus USFO+AVI^{12}, with a minimal effect on the parent adenovirus USFO. The maximum level of expression of packaging of USFO+AVI^{12} by COUP-TF expression was 400-fold.

Since our goal is to selectively repress adenovirus packaging using COUP-TF expression and

binding to specific packaging sequences, elimination COUP-TF repression of the adenovirus MLP was used to demonstrate its effect on viral packaging. A viable adenovirus mutant (termed USF-0; Reach et al. 1990) which contains mutations in the USF binding site was utilized. Binding studies showed that the USF-0 mutations disrupted the binding of COUP-TF to the MLP. Importantly, COUP-TF was not able to repress the USF-0 virus when tested for infectious virus yield *in vivo* and when MLP activity was analyzed *in vivo* in conjunction with COUP-TF overexpression (See Figure 9(c)). Importantly, COUP-TF expression had a minimal impact of viral late gene expression indicating the specific repression of packaging of USFO+AVI[12].

EXAMPLE 7

Repression of adenovirus packaging by the lac repressor. Figure 10C depicts a "designer" adenovirus vector whereby a binding site for the bacterial lac repressor is situated adjacent to and overlapping adenovirus packaging repeat AV. The lac repressor binding site is a perfectly symmetric sequence that binds lac repressor very tightly (Sadler et al. 1983). Lac repressor is a bacterial protein not expressed in eukaryotic cells. Eukaryotic cell, high level expression vectors were generated in our laboratory that express two forms of the lac repressor: 1) the wild type lac repressor, 2) the X86 mutant lac repressor which binds with 40-fold greater affinity to a lac site that the wild type lac repressor. Both forms of lac repressor carry epitope-tag (M45) at the amino-terminus for detection of protein expression in eukaryotic cells

by Western blot analysis using a monoclonal antibody against the epitope-tag (mAb M45; Obert et al. 1994). Fig. 11 shows a Western blot analysis of lac repressor expression in transfected 293 cells showing stable and high level expression of wild type and X86 lac repressors. Fig. 11 also shows a gel mobility shift assay using wild type and X86 Lac repressors expressed *in vivo* with a DNA probe containing the sequence shown in Fig. 10C. Stable DNA binding to the probe by both repressor forms is evident; specificity for Lac repressor is verified since: a) no binding is evident in cell extracts lacking Lac repressor, and b) the monoclonal antibody against Lac repressor alters the mobility (supershifts) the DNA-protein complex.

A recombinant adenovirus was generated that contains two copies of AV-VII + lac (Fig. 10C) in place of the adenovirus type 5 packaging domain (nt 194-814). The virus is viable and successfully propagated. AV-VII + lac viral DNA was cotransfected with the Lac repressor wild type high level expression vector (CMX + lac repressor) or with a control vector (CMX) into human 293 cells. Two days later, production of infectious virus was assayed. The results (Fig. 12) showed that lac repressor expression specifically repressed production of the "designer" virus AV-VII+lac. The maximum level of repression of packaging of AV-VII+lac by lac repressor expression was 20-fold.

EXAMPLE 8

The binding of P complex to A repeat sequences *in vitro* is dramatically reduced in the presence of ATP + $MgCl_2$. That is, the addition of 1 mM ATP + 2-10 mM

- $MgCl_2$ to a standard DNA binding reaction with an A repeat DNA probe results in near total elimination of the P complex binding activity. This effect is not observed when a non-hydrolizable analogue of ATP is used (gamma-S-ATP), thus ATP hydrolysis is involved in this process. P complex binds to AT-rich A repeat DNA sequences. An abundant cellular, nuclear protein that binds to such sequences is the TATA binding protein (TBP) which is a cellular transcription factor involved in transcription of cellular promoters. P complex binding is specifically competed using a known, high affinity TBP binding site (TATA box) which is supportive of the idea that P complex may contain TBP. P complex also binds to the adenovirus terminus to sequences 1-13. A panel of site-directed points mutations was made through this region to identify the binding site and it was found that all but one of the individual mutations did not reduce P complex binding, while combinations of multiple mutations reduced P complex binding 10-fold or greater. This type of binding pattern is consistent of a protein making interactions with the minor groove of the DNA, instead of the major groove of the DNA. It is known that TBP binds to the minor groove of DNA.

A protein complex containing TBP plus another protein termed TAF172 has been described (alternatively named TAF170; Timmers et al. 1992, Taggart et al. 1992). Both TBP and TAF172/170 are cloned (Hoffman et al., 1990, Kao et al. 1990, Knaap et al. 1997, Chicca et al. 1998). TAF172 has intrinsic ATP'ase activity and the TBP-TAF172 complex is displaced from DNA in the presence of ATP + $MgCl_2$, as found with P complex and A repeat binding (described above). A purification scheme, for P

complex activity has been developed (Fig. 5). P complex (identified by mobility shift assay with a DNA probe) and TBP-TAF172 complex (identified by Western blot using anti-TBP and anti-TAF172 antibodies) copurify through each column used with a final P compl3ex purification of ~1000-fold. Taken together, these results indicate that P complex contains TBP-TAF172.

LITERATURE CITED

Amalfitano, A., M.A. Hauser, H. Hu, D. Serra, C.R. Begy, and J.S. Chamberlain. 1998. Production and Characterization of Improved Adenovirus Vectors and with E1, and E3 Genes Deleted. Journal of Virology 72: 926-933.

Aoyama, A. and M. Hayashi. 1986. Synthesis of bacteriophage fX174 *in vitro*: mechanism of switch from DNA replication to DNA packaging. Cell 47: 99-106.

Beato, M., P. Herrlich and G. Schnetz. 1995. Steroid hormone receptors: many actors in search of a plot. Cell 83: 851-857.

Berkner, K.L., 1992 Curr. Topo. Micro. Immunol. 158:39-66.

Brough, D.E., A. Lizonova, C. Hsu, V.A. Kulesa and I. Kovesdi. 1996. A gene transfer vector-cell line system for complete functional complementation of adenovirus early regions E1 and E4. J. Virol. 70:6497-6501.

Chicca, J.J., D.T. Auble and B.F. Pugh. 1998. Cloning and biochemical characterization of TAF-172, a human homologue of yeast Motl. Mol. Cell. Biol. 18: 1701-1710.

Chou, J. and B. Roizman. 1989. Characterization of DNA sequence-common and sequence-specific proteins binding to cis-acting sites for cleavage of the terminal a sequence of the Herpes Simplex Virus 1 genome. J. Virol. 63: 1059-1068.

Connelly, S., T.A.G. Smith, G. Dhir, J.M. Gardner, M.G. Mehaffey, K.S. Zaret, A. McClelland and M. Kaleko. 1995. *In vivo* Gene Delivery and Expression of

Physiological Levels of Functional Human Factor VIII in Mice. Human Gene Therapy 6: 185-193.

Cooney, A. J., S. Y. Tsai, B. W. O'Malley and M.-J. Tsai. 1992. Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the vitaminD3, thyroid hormone, and retinoic acid receptors. Mol. Cell. Biol. 12: 4153-4163.

Dignam, J. D., R. M. Lebovitz and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11: 1475-1489.

Engelhardt, J.F., X.Ye, B. Doranz and J.M. Wilson. 1994. Ablation of E2 in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. Proc. Natl. Acad. Sci. USA 91:6196-6200.

Feinberg, A. P. and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132: 6-13.

Fredman, J. N., S. C. Pettit, M. S. Horwitz and J. A. Engler. 1991. Linker insertion mutations in the adenovirus preterminal protein that affect DNA replication activity in vivo and *in vitro*. J. Virol. 65: 4591-4597.

Freimuth, P. L. and H. S. Ginsberg. 1986. Codon insertion mutants of the adenovirus terminal protein. Proc. Nat. Acad. Sci. USA 83: 7816-7820.

Graeble, M. and P. Hearing. 1992. Cis and trans requirements for the selective packaging of adenovirus type 5 DNA. J. Virol. 66: 723-731.

Graham, F.L., J. Smiley, W. C. Russell and R. Nairn. 1977. Characteristics of a human cell line transformed by Dna from human adenovirus type 5. J. Gen. Virol. 36:59-74.

Haddada, H., T. Ragot, L. Cordier, M. T. Duffour and M. Perricaudet. 1993. Adenovirus interleukin-2 gene transfer into P815 tumor cells abrogates tumorigenicity and induces antitumoral immunityin mice. Human Gene Therapy 4:703-711.

Hardy, S., M. Kitamura, T. Harris-Stansil, Y. Dai and M.L. Phipps. 1997. Construction of adenovirus vectors through Cre-lox recombination. J. Virol. 71: 1842-1849.

Harris, M.P., S. Sutjipto, K.N. Wills, W. Hancock, D. Cornell, D.E. Johnson, R.J. Gregory, H.M. Shepard and D.C. Maneval. 1996. Adenovirus-mediated p53 gene transfer inhibits growth of human tumor cells expressing mutant p53 protein. Cancer Gene Ther. 3:121-130.

Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Molec. Biol. 26: 365-369.

Hoffman, A., E. Sinn, T. Yamamoto, J. Wang, A. Roy, M. Horikoshi and R.G. Roeder. 1990. Highly conserved core domain and unique N terminus with presumptive regulatory motifs in the human TATA factor (TFIID). Nature 346: 387-390.

Huang, M.M. and P. Hearing. 1989. The adenovirus early region 4 open reading frame 6/7 protein

regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. Genes & Development 3: 1699-1710.

Jaffe, H. A., C. Danel, G. Longenecker, M. Metzger, Y. Setoguchi, M. A. Rosenfeld, T. W. Gant, S.S. Thoreirsson, L.D. Stratford-Perricaudet and M. Perricaudet. 1992. Adenovirus-mediated in vivo gene transfer and expression in normal rate liver. Nat. Genet. 5: 372-378.

Jobe, A. and S. Bourgeois. 1972. The *lac* repressor-operator interaction. VII. A repressor with unique binding properties: the X86 repressor. J. Mol. Biol. 72: 139-152.

Jones, N. and T. Shenk. 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell 17: 683-689.

Kao, C.C., P.M. Lieverman, M.C. Schmidt, Q. Zhou, R. Pei and A.J. berk. 1990. Cloning of a transcriptionally active human TATA binding factor. Science 248: 1646-1650.

Kemble, G. W. and E. S. Mocarski. 1989. A host cell protein binds to a highly conserved sequence element (pac-2) within the cytomegalovirus a sequence. J. Virol. 63: 4715-4728.

Krougliak, V. and F.L. Graham. 1995. Development of cell lines capable of complementing E1, E4, and protein IX defective adenovirus type 5 mutants. Hum. Gene Ther. 6: 1575-1586.

Kunkel, T.A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82:488-492.

Ladias, J. A. and S. K. Karathanasis. 1991. Regulation of the apolipoprotein AI gene by ARP-1, a novel member of the steroid receptor superfamily. Science 251: 561-565.

Obert, S., R.J. O'Connor, S. Schmid and P. Hearing. 1994. The adenovirus E4-6/7 protein transactivates the E2 promoter by inducing dimerization of a heteromeric E2F complex. Mol. Cell. Biol. 14: 1333-1346.

Parks, R.J., L. Chen, M. Anton, U. Sankar, M.A. Rudnicki and F.L. Graham. 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. Proc. Nat'l Acad. Sci. USA 93: 13565-13570.

Qiu, Y., F.A. Pereira, F.J. DeMayo, J.P. Lydon, S.Y. Tsai and M.J. Tsai. 1997. Null mutation of mCOUP-TFI results in defects in morphogenesis of the glossopharyngeal ganglion, axonal projection, and arborization. Genes Dev. 11: 1925-1937.

Reach, M., L.E. Babiss and C.S.H. Young. 1990. The upstream factor binding site is not essential for activation from the adenovirus major late promoter. J. Virol. 64: 5851-5860.

Rosenfeld, M.A., K. Yoshimura, B.C. Trapnell, K. Yoneyama, E.R. Rosenthal, W. Dalemans, M. Fukayama, J. Bargon, L.E. Stier, L. Stratford-Perricaudet, M. Perricaudet, W.B. Gudgeon, A. Pavirani, J.P. Lecocq and R.G. Crystal. 1992. *In Vivo* Transfer of the Human Cystic Fibrosis Transmembrane Conductance Regulator Gene to the Airway Epithelium. Cell 68: 143-155.

Rosenfeld, P. J., E. O'Neill, R. J. Wides and T. J. Kelly. 1987. Sequence-specific interactions

between cellular DNA-binding proteins and the adenovirus origin of DNA replication. Mol. Cell. Biol. 7: 875-886.

Sadler, J.R., H. Sasmor and J.L. Betz. 1983. A perfectly symmetric *lac* operator binds *lac* repressor very tightly. Proc. Nat. Acad. Sci. USA 80: 6785-6789.

Sagami, I., S. Y. Tsai, H. Wang, M. J. Tsai and B. W. O'Malley 1986. Identification of two factors required for transcription of the ovalbumin gene. Mol. Cell. Biol. 6: 4259-4267.

Sawadogo, M. and R.G. Roeder. 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell 43: 165-175.

Schmid, S. I. and P. Hearing. 1997. Bipartite structure and functional independence of adenovirus type 5 packaging elements. J. Virol. 71: 3375-3384.

Schmid, S. I. and P. Hearing. 1998. Cellular Components interact with adenovirus types 5 minimal DNA packaging domains. J. Virol. 72: 6339-6347.

Stow, N. D. 1981. Cloning a DNA fragment from the left-hand terminus of the adenovirus type 2 genome and its use in site-directed mutagenesis. J. Virol. 37: 171-180.

Strubin, M. and Struhl, K. 1992. Yeast and human TFIID with altered DNA binding specificity for TATA elements. Cell 68: 721-730.

Taggart, A.K.P., T.S. Fisher and B.F. Pugh. 1992. The TATA-binding protein and associated factors are components of pol III transcription factor TFIIIB. Cell 71: 1015-1028.

Timmers, H.T.M., R.E. Meyers, and P.A. Sharp. 1992. Composition of transcription factor B-TFIID. Proc. Nat. Acad. Sci. USA 89: 8140-8144.

Tsai, S. Y. and M. J. Tsai. 1997. Chicken ovalbumin upstream promoter-transcription factors (COUP-TFs): coming of age. Endocrine Reviews 18: 229-240.

van der Knaap, J.A., J.W. Borst, P.C. van der Vleit, R. Gentz and H.T.M. Timmers. 1997. Cloning of the cDNA for the TATA binding protein-associated factorII 170 subunit of transcription factor B-TFIID reveals homology to global transcription regulators in yeast and *Drosophila*. Proc. Nat. Acad. Sci. USA 94: 11827-11832.

Vliet, P. C. v. d. 1995. Adenovirus DNA replication. Curr. Top. Microbiol. Immunol. 199/II: 1-30.

Wang, L.-H., S. Y. Tsai, R. G. Cook, W. G. Beattie, M.J. Tsai and B. W. O'Malley. 1989. COUP transcription factor is a member of the steroid receptor superfamily. Nature 340: 163-166.

Weber, J. M., C. V. Dery, M. A. Mirza and J. Horvath. 1985. Adenovirus DNA synthesis is coupled to virus assembly. Virology 140: 351-359.

Williams, J. F., M. Gharpure, S. Ustacelebi and S. McDonald. 1971. Isolation of temperature-sensitive mutants of adenovirus type 5. J. Gen. Virol. 11: 95-101.

Wills et al., 1994. Human Gene Therapy 5:1079-188.

Yang, Y., F.A. Nunes, K. Berencsi, E.E. Furth, E. Gonczol and J.M. Wilson. 1994. Cellular immunity to

viral antigens limit E1-deleted adenoviruses for gene therapy. Proc. Natl. Acad. Sci. USA 91:4407-4411.

Yang, Y., F.A. Nunes, K. Berencsi, J.F. Engelhardt and J.M. Wilson. 1994A. Inactivation of E2 in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. Nat. Genet. 7:362-369.